Marek's disease herpesvirus vaccines integrate into chicken host chromosomes yet lack a virus-host phenotype associated with oncogenic transformation

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ABSTRACT

Marek's disease (MD) is a lymphotropic and oncogenic disease of chickens that can lead to death in susceptible and unvaccinated host birds. The causative pathogen, MD virus (MDV), a highly oncogenic alphaherpesvirus, integrates into host genome near the telomeres. MD occurrence is controlled across the globe by biosecurity, selective breeding for enhanced MD genetic resistance, and widespread vaccination of flocks using attenuated serotype 1 MDV or other serotypes. Despite over 40 years of usage, the specific mechanism(s) of MD vaccine-related immunity and anti-tumor effects are not known. Here we investigated the cytogenetic interactions of commonly used MD vaccine strains of all three serotypes (HVT, SB-1, and Rispens) with the host to determine if all were equally capable of host genome integration. We also studied the dynamic profiles of chromosomal association and integration of the three vaccine strains, a first for MD vaccine research. Our cytogenetic data provide evidence that all three MD vaccine strains tested integrate in the chicken host genome as early as 1 day after vaccination similar to oncogenic strains. However, a specific, transformation-associated virus-host phenotype observed for oncogenic viruses is not established. Our results collectively provide an updated model of MD vaccine-host genome interaction and an improved understanding of the possible mechanisms of vaccinal immunity. Physical integration of the oncogenic MDV genome into host chromosomes along with cessation of viral replication appears to have joint signification in MDV's ability to induce oncogenic transformation. Whereas for MD vaccine serotypes, a sustained viral replication stage and lack of the chromosome-integrated only stage were shared traits during early infection.

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1. Introduction

Marek's disease (MD) is a lymphotropic disease of chickens characterized by fatal lymphoma development in the visceral organs, paralysis and blindness in susceptible birds. The causative alphaherpesvirus [1,2], Marek's disease virus (MDV aka gallid herpesvirus type 2 or serotype 1), is known to integrate into chicken telomeres [3–5]. The MDV genome contains homologous host genes and telomeric repeats, which were presumably acquired through recombination and/or integration and reemergence events during the course of the virus' evolutionary history [6–8]. As a herpesvirus, the life cycle of virulent strains involves transitioning from a stage of cytolytic replication in the episomal form to a latent stage, by which MDV evades host immune responses [9–14]. Furthermore, latent host CD4+ T lymphocytes become transformed after serotype-1 oncogenic MDV infection, between 14 and 21 days post-infection (dpi), resulting in lymphomas [15–19]. Telomeric integration of MDV is temporally related to latency [4,5]. Furthermore, latently-infected MD cell lines consist of primarily host genome integrated MDV, rather than extra-chromosomal viral DNA [3,20]. MDV telomeric repeats facilitate integration into chicken telomeres [21,22], although the precise mechanism of integration is not fully known. The viral *Meq*

oncogene and viral telomerase (vTR) gene, among others, play critical roles in host cell transformation [23–25]. Interestingly, a *Meq*-deleted (ΔMeq) MDV strain that does not induce MD lymphomas *in vivo* [26] can integrate into the chicken genome, but lacks a transformation-coupled viral-host phenotype in lymphocytes [5]. Osterrieder and colleagues presented experimental data suggesting that vTR is involved in telomeric integration of MDV [9].

MD can be controlled by selective breeding for increase genetic resistance, however, in commercial production systems, the primary control method is widespread vaccination [16]. An effective MD vaccine, the related herpesvirus of turkey (HVT; meleagrid herpesvirus type 1 aka serotype 3) [27,28], was first employed in the early 1970s. Field strain virulency increased (very virulent (vv) to vv+ ratings) through the late 1970s resulting in more outbreaks until bivalent vaccine [SB-1 (gallid herpesvirus type 3 or serotype 2) + HVT] and, later, the attenuated serotype 1 Rispens/CV1988 vaccine were employed [29,30]. The Rispens vaccine provides the highest level of protection against vv+ MDV, although its protective efficiency varies with bird major histocompatibility complex (MHC) haplotype [31]. These non-oncogenic, vaccine strains have been detected in a latent form *in vivo*, but do not induce MD lymphomas [32]. Current vaccination strategies do not elicit sterilizing immunity [18]. The specific mechanisms of vaccine-related immunity are not known, although reduced growth rate of MDV [33], increased late reactivity of peripheral blood lymphocytes to mitogens (in the case of HVT) [34] and prevention of immunosuppressive effect in the host [35] may contribute in addition to vaccine strain(s) interference with the infection process of oncogenic MDV in T and B lymphocytes [36,37]. However, supplementary mechanisms are certainly involved [9,15] and an explanation for the anti-tumor effect of vaccination has not yet been established. Furthermore, the underlying features of the vaccinal protective mechanism undoubtedly vary among the vaccine serotypes.

Although the genomes of oncogenic and vaccine strains are highly similar, key differences exist [38] (*see Table 1 p.2*). A shared aspect among serotypes is the presence of telomeric repeats between the inverted repeats long (IR_L)/short (IR_S) and terminal repeats long (TR_L)/short (TR_S) of the viruses. The presence of telomeric repeats in both oncogenic and vaccine MDV strains suggests that the vaccines could be capable of integrating into telomeres of host lymphocytes, despite differences in or the absence of, multiple disease-associated genes (*Meq*, vTR, vIL8, etc.) [39]. Our central hypothesis is that MD vaccines integrate into the chicken genome at the telomeres. This vaccinal interaction with the host genome, in addition to various other behaviors that contribute to the vaccinal protective mechanism, may disrupt aspects of infection, latency, and/or the transformation cycle of the oncogenic MDV strain(s). Here we sought to test our hypothesis by investigating interactions of MD vaccines with the host at the level of the genome (*i.e.*, study virus chromosomal association and integration profiles) via cytogenetic methods.

2. Materials and methods

2.1. Genetic lines and vaccination/challenge

Experimental birds were progeny of a cross between two highly inbred, MD susceptible lines 7_2 and $15I_5$ from the USDA, ARS, Avian Disease and Oncology Laboratory (ADOL), cared for under approved animal care protocols by trained staff. All institutional (ADOL Institutional Animal Care and Use Committee) and national (USA National Research Council of the National Academies) guidelines for the appropriate care and use of laboratory animals were closely followed throughout the experiments. One week post-hatch, F_1 chicks were intra-abdominally vaccinated with 5000 pfu of HVT, SB-1, or Rispens alone, or challenged with GA oncogenic MDV alone or received no treatment. Chicks were hatched and maintained in Horsfall-Bauer isolation chambers at the USDA facility.

2.2. Spleen sample collection and processing

Mitotic chromosome preparations were harvested from spleen samples collected at 1, 4, 7, 14 and 21 days post-infection (dpi). Spleen cell suspensions were treated with 100 μl of colcemid (10 μg/ml, Invitrogen, Waltham, MA, USA) in cell culture medium for 30–60 min. Single cell suspensions were generated from each spleen and exposed to 0.56% KCl hypotonic solution (30 min) and then fixed in 3:1 methanol and glacial acetic acid (several fixes over one to three days) according to standard procedures. The chromosome spreads were applied to slides using the air-dry method after the third fixative change, as described by Delany et al. [40]. All chromosome slides were stored according to Delany et al. [40] until further use in FISH experiments, where they were left at room temperature in a slide box for at least 24 h to allow thawing and for condensation to be absorbed by fresh desiccant.

2.3. MDV-specific probes and probe labeling

The DNA probe utilized for hybridization to MDV genome was an Md11 bacterial artificial chromosome (BAC) (Md11gDc1.2 [7]). The probes were labeled using nick translation with Digoxigenin (DIG) dUTP through a DIG Nick Translation kit (Roche Applied Science, Indianapolis, IN, USA). An anti-digoxigenin secondary antibody (Roche Applied Science) conjugated with Fluorescein isothiocyanate (FITC, green) was then applied. Due to the presence of chicken telomeric repeats ($TTAGGG_n$) in the Md11 BAC probe, telomeric DNA sequence in the chicken genome was blocked with approximately 280 ng/uL of custom telomere oligonucleotide ($TTAGGG_7$), also called "cold telo."

2.4. Fluorescence in situ hybridization (FISH)

Slide hybridization was completed by standard FISH procedures as described by Delany et al. [40] with adaptations for labeling MDV; (1) the Md11 BAC probe was applied; (2) cold telo was utilized to block host telomeric DNA. The preparations were counterstained with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and 4′,6-diamidino-2-phenylindole (DAPI, Vector Laboratories) diluted 1:9 (DAPI:VS). Prior to analysis, the slides were stored flat at 4 °C and image capture occurred within 24 h of FISH and DAPI counterstaining.

2.5. Cytogenetic analysis

Metaphase and interphase cell images were collected using an Olympus BX41 epifluorescence microscope equipped with an automatic filter wheel (Chroma Technology 82000, DAPI/fluorescein isothiocyanate/tetra methyl rhodamine isothiocyanate filter set), X-Cite 120 Series metal-halide fiber optic lamp, and Applied Imaging software (CytoVision 7.4 GENUS, Leica Biosystems, Buffalo Grove, IL, USA). Between 10 and 80 spleen cell images were analyzed per sample (representing an individual spleen from a bird) in each FISH experiment. Negative control (no treatment) samples were incorporated in most MDV FISH experiments to ensure that the Md11 BAC probe was hybridizing specifically to the MDV genome, as indicated by the absence of FITC signals from all terminal and interstitial telomeres, as described by Robinson et al. [4]. All captured mitotic metaphase cells were categorized as one of four cytogenetic viral phenotypes as previously described [4,38] and

shown in Fig. 1: null (no signals detected), chromosome-associated (dispersed signals over and around the chromosomes); chromosome-associated and telomere-integrated (associated signals along with distinct, punctate and bright signal(s)); or telomere-integrated only (only the distinct telomeric signals detected).

2.6. Statistical analysis

The mean values of metaphase cells with the specific phenotypes were evaluated by the multiple comparison of means test in R (*R Core Team, version 3.2.1, 2015*) [41] across timepoints and treatment groups to establish statistically significant differences.
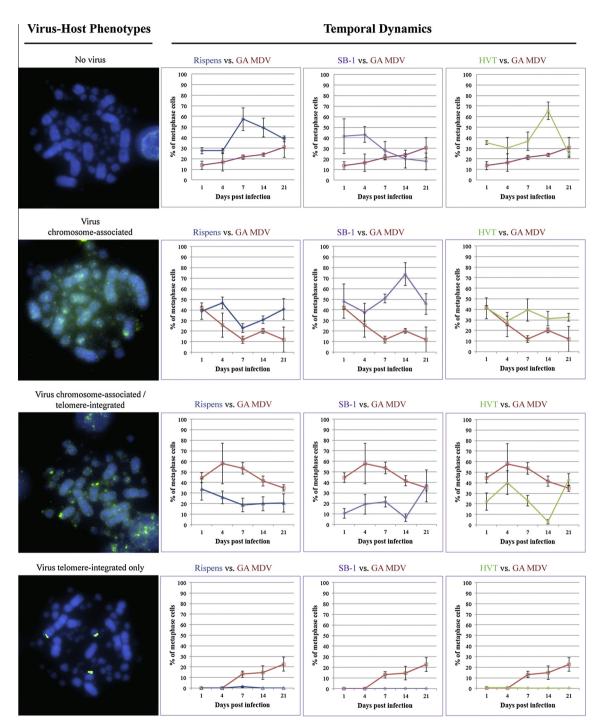

Fig. 1. Temporal dynamics of MDV vaccine-host genome interactions in spleen mitotic cell populations: patterns of virus-host phenotypes over time. Representative FISH images of the cytogenetic phenotypes observed, which signify the status of the herpesvirus with regard to the chicken host genome in mitotic cells of the spleen, are to the left of the corresponding data for those phenotypes. The null phenotype lacks viral FISH signals (FITC, green) around the chromosomes (DAPI, blue). The viral chromosome-associated phenotype is defined by diffuse viral FISH signals surrounding the host chromosomes while the chromosome-associated/telomere-integrated phenotype consists of both the diffuse associated signals and bright, punctate integrated-virus signals at the telomeres. The telomere-integrated only viral phenotype is exclusively comprised of distinct, punctate FISH signals at the telomeres. For the corresponding phenotype-data graphs, the lines represent the mean percentage of mitotically dividing cells with a given phenotype ± SEM for 3 birds per treatment group across all early timepoints (1–21 dpi). The oncogenic MDV (GA; red) phenotype data is compared to the data for each MD vaccine (Rispens; blue, SB-1; purple, HVT; green), respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As described by Robinson et al. [5], raw count data were transformed using a logit transformation (logit (k/n) = ln {k + 1/n − k + 1}), in which k out of n cells were observed for any given viral phenotype within a bird and timepoint. The logit or logarithm of the odds transformation is applied to fit categorical data in the form of proportions to a linear model. The normalized data were then analyzed by two-factor ANOVA and, subsequently, Tukey's multiple comparisons of means with a 95% family-wise confidence interval. The Tukey's method of multiple comparison analysis tests all pairwise differences between groups, even when unequal group sizes exist, and reduces the probability of type I error through use of the q statistic. The effects of MDV strain and days post infection/vaccination on phenotype were calculated. The count of metaphase cells identified within each phenotype was the dependent variable in ANOVA analysis.

3. Results

We investigated virus-host chromosomal association and integration profiles of three MDV serotypes (three vaccines, one oncogenic strain) in three animals per treatment group at each of five timepoints. As is commonly observed with MD research results, inter-individual variation within a treatment group was evident for virus-host phenotypes despite identical host genetic backgrounds, tissue sampled, bird ages, timing of vaccination/challenge and sample conditions, including vaccine/challenge dosages and environmental conditions.

3.1. MD vaccines integrate in the chicken host genome

Our results showed evidence of viral integration, *i.e.*, punctate viral (FITC, green) signals at the telomeres of mitotic metaphase chromosomes of spleen cells, in the MDV challenged (GA) birds, and also in all MD vaccinated (Rispens, SB-1, HVT) birds. The appearance (distinct, bright, and frequently doublet sister chromatid signals) and location of these FITC signals indicate that they represent telomere-integrated MDV genome as a long concatemer. For all treatment groups (excluding the negative control), telomere-integrated MDV signals were detected in spleen cells immediately post-treatment (1 dpi) through to 21 dpi (Fig. 1). Although all MDV challenged or vaccinated birds presented with telomere-integrated virus, the frequency of MDV integration displayed a notable degree of inter-individual variation within and, more significantly, between treatment groups, consistent with prior research [4,5]. Across all timepoints, oncogenic MDV (GA) integrated into the host telomeres in a higher percentage of cells as compared the MD vaccines. Furthermore, the GA-challenged birds exhibited both the MDV chromosome-associated/telomere-integrated phenotype and the telomere-integrated only phenotype. However, the MD vaccinated-birds lacked the telomere-integrated only phenotype between 1 and 21 dpi in the spleen (Table 1), except in rare instances (single cell from a Rispens-vaccinated sample at 7 dpi, and from HVT-vaccinated samples at 1 and 4 dpi).

3.2. Cytogenetic interactions with the host genome

3.2.1. Rispens vaccine versus oncogenic MDV

The Rispens vaccination treatment group had a higher mean percentage of cells with the MDV-null and MDV-chromosome-associated phenotypes across all time points, with the exception of 1 dpi for the latter phenotype, as compared to the GA-challenged group (Fig. 1). The Rispens-vaccinated birds also demonstrated a lower mean percentage of cells with the MDV-chromosome-associated/telomere-integrated phenotype at all timepoints. These results could reflect a longer cytolytic stage for the non-oncogenic Rispens virus as compared to oncogenic/virulent MDV, perhaps due to inability of the vaccine-virus to efficiently or entirely transition into latency in host cells. Most changes in the percentage of cells with the MDV-chromosome-associated phenotype, likely representative of viral lytic replication, were reflected by inverse changes in the percentage of cells with the MDV-null phenotype at the same time points (while the transi-

Table 1
A comparison of viral-host cytogenetic interactions for oncogenic and vaccine MDV strains in the splenic cell populations during early infection.

Days post infection	Virus type	Phenotype			
		Null	Chromosome-associated	Chromosome-associated/ telomere-integrated	Telomere-integrated only
1	GA	13.7 ± 6.7	41.8 ± 2.7	44.5 ± 8.1	0 ± 0
1	Rispens	27.5 ± 5	38.8 ± 13.6	33.7 ± 18	0 ± 0
1	SB-1	41.4 ± 28.6	48.1 ± 27.9	10.5 ± 7.8	0 ± 0
1	HVT	35.4 ± 3	41.1 ± 17.2	22.1 ± 14.2	0.7 ± 1.2
4	GA	16.5 ± 14.3	25.6 ± 20	57.9 ± 33.3	0 ± 0
4	Rispens	27.6 ± 3.6	46.4 ± 9.5	25.9 ± 10.5	0 ± 0
4	SB-1	43.1 ± 12.9	37.7 ± 15	19.2 ± 16.2	0 ± 0
4	HVT	30.3 ± 17.5	29 ± 7.8	40.1 ± 19.4	0.5 ± 0.9
7	GA	21.5 ± 3.6	11.9 ± 5.8	*53.6**b* ± 9.5*	*13.1**b* ± 5.3*
7	Rispens	57.3 ± 18.4	22.8 ± 7.1	18.7 ± 11.4	1.3 ± 1.1
7	SB-1	27.8 ± 15.1	50.8 ± 7.2	21.5 ± 8.2	0 ± 0
7	HVT	36.7 ± 15.3	39.5 ± 18.4	23.1 ± 8.7	0 ± 0
14	GA	23.8 ± 2.7	20.1 ± 3.9	41.5 ± 8.2	14.7 ± 11.2
14	Rispens	49.3 ± 15.3	30.8 ± 6.1	19.9 ± 11.6	0 ± 0
14	SB-1	19.9 ± 14.7	*73.7**b* ± 18.8*	6.4 ± 6	0 ± 0
14	HVT	65.6 ± 14.4	31.3 ± 12	**3.1*a ± 3.7**	0 ± 0
21	GA	30.7 ± 16.4	12 ± 20.7	34.8 ± 5.1	*22.7**b* ± 11.3*
21	Rispens	38.6 ± 4.5	40.9 ± 14.7	20.6 ± 14.7	0 ± 0
21	SB-1	17.8 ± 13.9	45.6 ± 17.1	36.7 ± 26.5	0 ± 0
21	HVT	24.7 ± 3.5	32.5 ± 6.6	42.8 ± 9.8	0 ± 0

The mean percentages ± standard deviation of 3 birds with given virus-host cytogenetic phenotypes for each of the treatment groups (oncogenic strain: GA, non-oncogenic vaccine strains: Rispens, SB-1, HVT) over five timepoints (1–21 dpi). See Methods and Results for virus-host phenotype explanations. The values that were found to be statistically significant within a viral phenotype for a treatment group (MDV strain) over time are indicated in bold font and a "*a*" symbol (p < 0.05). The values that were statistically significantly different as compared to the other treatment groups (MDV strains) within a timepoint and within a viral phenotype are indicated in italic font and a "*b*" symbol (p < 0.05).

tional viral phenotype showed no detectable shifts). This can be most prominently observed at 7 dpi, whereupon the mean percentage of cells with the null phenotype increases as the percentage of MDV-chromosome-associated phenotype declines compared to 4 dpi (Fig. 1). Thus, reduction in replicating Rispens virus in the spleen may be due to viral clearing by the host immune responses, rather than explained by the Rispens vaccine-virus transitioning into latency during early infection (1–21 dpi). Conversely, GA MDV shows an increasing population of cells with the telomere-integrated only phenotype as the percentages of cells with the chromosomes-associated phenotypes decline between 4 and 21 dpi.

3.2.2. SB-1 vaccine versus oncogenic MDV

SB-1 vaccine treatment showed a unique trend for the MDV null phenotype as compared to the other vaccine types (Rispens or HVT). The mean percentage of cells with this phenotype for the vaccinated birds was initially higher than that of the challenged birds as was the circumstance for the other two vaccinated groups. However, the null phenotype declines steadily from 4 to 21 dpi and drops below the mean percentage observed for the GA-challenged birds by 14 dpi. The distinctly low percentage of MDV-null cells at the later timepoints (14 and 21 dpi) may indicate that the SB-1 vaccine-virus is able to establish a particularly prolonged cytolytic replication stage in the host and/or better evade host immune responses during early infection.

Similar to the other vaccination groups, the SB-1 vaccinated birds consistently demonstrated a higher mean percentage of cells with the MDV chromosome-associated phenotype, which appears to represent a cytolytic stage of infection, as compared to the GA-challenged birds through all timepoints of early vaccination/infection (1–21 dpi). There was also a notable increase in cells with this viral phenotype at 14 dpi, which was significantly different ($p < 0.05$) from the mean percentages across timepoints and within the phenotype for SB-1 vaccinated birds. The significant increase in the percentage of cells with the MDV chromosome-associated phenotype observed at 14 dpi was mirrored by a decrease in the MDV chromosome-associated and telomere-integrated phenotype, while the MDV null phenotype continued on a steady downward trend.

3.2.3. HVT vaccine versus oncogenic MDV

The HVT-vaccinated treatment group demonstrated a higher percentage of cells in the spleen with the MDV chromosome-associated phenotype, representing cytolytic replication in host nuclei, as compared to the oncogenic MDV-challenged group at all timepoint except 1 dpi. This may represent a higher and more sustained lytic infection by the HVT vaccine-virus as compared to oncogenic MDV. The percentage of cells with the null phenotype (no virus detected) was also higher in the vaccinated birds until 21 dpi. Conversely, the percentage of dividing cells with the MDV chromosome-associated and telomere-integrated phenotype was lower for the HVT treatment group as compared to the challenged group until 21 dpi.

At 14 dpi, a significant ($p < 0.05$) decrease in the mean percentage of mitotic cells with the transitional (MDV chromosome-associated and telomere-integrated) phenotype was observed in HVT-vaccinated birds. The dramatic reduction in this viral phenotype was accompanied by a notable increase in the MDV null phenotype (no MDV detected). The large shift in MD vaccine presence within the spleen may be explained by a decline in MDV-infected cells following host adaptive immune response and infected host cell targeting. It is interesting that there is no significant change in the MDV chromosome-associated only phenotype at 14 dpi. The subsequent increase in the transitional phenotype at 21 dpi may indicate viral infection "rebound" in the spleen, perhaps due to a failure of the immune response to completely clear the virus. Interestingly, the mean percentage of mitotically dividing cells with the transitional phenotype in HVT-vaccinated birds surpasses that of the GA-challenged birds, while the percentage of cells with the MDV null phenotype in the vaccinated group falls below that of the challenged group, at 21 dpi.

4. Discussion

Prior research has shown [4,5] that the MDV telomere-integrated only phenotype is strongly associated with the timing of viral latency and host cell transformation in birds infected with oncogenic MDV strains. Furthermore, >90% of mitotically dividing cells in MDV-induced lymphomas exhibit the MDV telomere-integrated only phenotype [5]. The MDV chromosome-associated/telomere-integrated phenotype may represent a transitional phase of the viral life cycle from lytic replication to latency (for most MDV strains) and transformation (in the case of oncogenic MDV). However, it is not yet known if this transitional virus-host phenotype presented with the MDV chromosome-associated phenotype at an earlier timepoint or established the transitional phenotype upon initial MDV infection. Rarely, T lymphocytes infected with latent MDV will undergo oncogenic transformation and, among those cells, a few will establish as tumors (i.e., T cell lymphomas in the visceral organs). The telomere integrated-only MDV phenotype likely represents a "transformation susceptible" latently-infected host cell that is expanding [3,4], which would explain the absence of this viral phenotype with the non-oncogenic MD vaccines. Furthermore, several MDV genes, such as Meq and vIL8, in the Rispens/CVI988 genome differ from those in the oncogenic serotype 1 MDV and these variations likely contribute to the attenuation of the vaccine-virus, which includes loss of the cellular transformation capacity [38,42]. The absence of the same transformation-associated genes in the SB-1 and HVT genomes also explains their inability to induce lymphomas [43,44].

In this study, all vaccinated birds showed the viral chromosome-associated/telomere-integrated phenotype, signifying that MD vaccine-viruses are capable of integrating into host chromosomes. This finding was interesting considering that although the viral telomeric repeats are intact within all vaccine genomes, the Meq and vTR genes, which contribute to oncogenic MDV integration, are absent from SB-1 and HVT genomes [43–46], and in the Rispens genome a functional copy of the Meq oncogene is lacking [19,38,42]. The absence of the MDV telomere-integrated only phenotype in dividing splenic cell populations (largely T and B lymphocytes, monocytes, macrophages, etc.) of MD-vaccinated birds was unsurprising given that the vaccines do not induce MD lymphomas. A ΔMeq MDV strain, which does not induce MD lymphomas [47], also did not demonstrate the telomere-integrated only virus-host phenotype in host immune organs [5]. However, it is interesting that the vaccines strains are all capable of telomeric integration, even during early infection, but fail to transition into the telomere-integrated only phase.

MD vaccines were found in numerous studies to replicate during early infection (1–21 dpi) and at later timepoints post-vaccination (up to 56 dpi) in various immune tissues (including spleen) [10,35,48,49], reduce oncogenic MDV viral load in vivo [49], and horizontally transmit through shed feather dander [48]. A distinct feature of all MD vaccine strains, demonstrated by our data, was a prolonged and elevated incidence of the MDV chromosome-associated phenotype in the spleen during early infection (up to 21 dpi), which has been correlated with the timing of lytic replication [4,5,11]. Unsurprisingly, variations in and/or the absence of transformation-associated MDV genes in the MD vaccines' genomes [42–44] did not impact the cytolytic replication abil-

ities of the vaccine strains. In fact, several genes, such as vTR and *Meq*, have been implicated in serotype 1 MDV lymphoma development, while their expression does not influence MDV replication *in vivo* [25,47]. The prolonged cytolytic stage observed in the MD-vaccinated birds may be, in part, explained by an inability of the vaccine strains to efficiently transition into latency. A reduced ability to establish latency has been suggested for the ΔMeq MDV strain as well [47]. It may be that the prolonged replication of the MD vaccines in host immune tissues leads to competitive exclusion against virulent/oncogenic MDV, contributing to the protective mechanism [50].

We have repeatedly employed molecular cytogenetics to study MDV behavior, and observed early (1 dpi) viral replication [4,5]. Our current data indicate the presence of replicating oncogenic MDV or vaccine-virus in host lymphoid tissue as early as 1 dpi, contrasting with studies that first detected vaccine DNA at 3 dpi in spleen [10]. The difference may be attributable in part to administration, *i.e.*, subcutaneous versus intra-abdominal, and the different resolving powers of single-cell cytogenetic versus polymerase chain reaction (PCR)-based detection. There is undoubtedly variation among the MD vaccine-virus serotypes, in terms of timing, duration, productivity and perhaps host immune tissue targeted during viral replication, in addition to shared differences from oncogenic MDV. However, it is clear from both cytogenetic and PCR-based data that there is a productive vaccine-virus lytic replication stage in vaccinated, yet unchallenged, susceptible chickens.

A prolonged and increased cytolytic replication stage in lymphoid tissues has been observed for very virulent (vv) and very virulent+ (vv+) MDV pathotypes as compared to virulent (v) MDV by molecular methods, along with other key differences in pathogenesis [51,52]. Investigations of Rispens, SB-1 and HVT indicated that MD vaccine lytic replication, specifically viral load during the timing of replication as detected by PCR-based techniques, was reduced as compared to virulent/oncogenic MDV challenged birds in the same immune tissues (splenic, thymic and bursal) [53–55]. It has also been established that attenuation of serotype 1 MDV (*i.e.*, Rispens vaccine development) is correlated with decreased replication ability as well as the loss of oncogenic transformation capacity [56]. This seems to suggest a positive correlation between MDV strain virulency and productivity of the cytolytic replication. Our molecular cytogenetic results indicate a prolonged and augmented viral replication stage of the three MD vaccine strains in splenic cell populations of highly susceptible birds. There are key differences in the meaning of 'viral load' as measured by PCR-based versus cytogenetic methods; namely, that the PCR-based viral load indicates a quantity of viral genomes per unit or volume of cells, whereas the cytogenetic load indicates a quantity of cells with replicating viral genomes. Thus, the PCR-based and cytogenetic data on MD vaccine versus oncogenic MDV replication may demonstrate both a higher percent of host splenic cells maintaining lytic MD vaccine replication (as detected by cytogenetic methods) and a vaccine strain replication that is not as productive in lytically-infected cells, resulting in a quantifiably lower vaccine-virus load (as detected by PCR-based methods). Additionally, molecular cytogenetic methods provide an enhanced sensitivity for the detection of lytically replicating, yet low production level, virus as compared to PCR quantification methods.

A remaining important question is whether the MD vaccines innately transition to viral latency in host birds and how vaccine serotype in addition to host MHC genotype, age and/or immune status impact the vaccine strain behavior among the immune organs (spleen, bursa of Fabricius and thymus). There have been indications of latent HVT in the spleen at 70 dpi and in all lymphoid tissues (thymus, spleen and bursa) at 105 dpi, however this finding did not exclude the possibility of vaccine-virus latency during earlier timepoints after vaccination [57]. Furthermore, latent,

and predominantly linear form, HVT and MDV genomes were detected within a non-producer T-lymphoblastoid cell line developed from the spleen of an HVT-vaccinated chicken [58]. Their results also indicated that latent viral genome replication, or transmission to daughter cells, exploits host cell replication factors rather than viral replication factors, which strongly suggested integrated-virus genomic DNA in latently-infected cells. As mentioned above, the detection of the transitional virus-host phenotype and the absence of the telomere-integrated only phenotype in the spleen of MD-vaccinated birds suggest that the vaccine-viruses do not efficiently transition to latency during early infection compared to pathogenic MDV. An investigation of lymphoid tissue of vaccinated chickens from later timepoints (>21 dpi) may help elucidate the temporal nature of MD vaccine strain latency.

Based on our cytogenetic results, it is evident that the MD vaccines maintain a prolonged lytic replication in host birds, and the percentage of lytically-infected cells in MD-vaccinated birds is higher than in birds challenged with oncogenic MDV. The MD vaccines also integrate at chicken host telomeres, although in a lower percentage of cells as compared to oncogenic MDV. Interestingly, the vaccines are similar to ΔMeq MDV [5] in behavior as both do not lead to the integrated-only viral phenotype, which is a hallmark of oncogenic MDV latency and transformation, in host spleen cells. The absence of a host cell population with integrated-MDV only in vaccinated birds may be useful to elucidate the vaccines' inability to establish latency during early infection and transform host cells. Physical integration of the oncogenic MDV genome into host telomeres along with cessation of viral replication appear to be significant in MDV's ability to induce disease symptoms and oncogenic transformation in the host. This updated model of oncogenic-MDV versus MD-vaccine behavior offers an improved understanding of the mechanisms of vaccinal immunity and should contribute to future MD vaccine research and development.

Ethical standards

The authors declare that all experiments performed in this study comply with the current laws of the United States of America. All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the guidelines set forth by the USDA-ADOL Institutional Animal Care and Use Committee.

Conflict of interest

None.

Acknowledgements

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